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**AN INVESTIGATION INTO THE OCCURRENCE OF
BATRACHOCHYTRIUM DENDROBATIDIS INFECTION IN
PLETHODONTID SALAMANDER COMMUNITIES OF ROBINSON
FOREST**

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AN INVESTIGATION INTO THE OCCURRENCE OF *BATRACHOCHYTRIUM DENDROBATIDIS*
INFECTION IN PLETHODONTID SALAMANDER COMMUNITIES OF ROBINSON FOREST

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in the College of Agriculture at the University of Kentucky

By

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Lexington, Kentucky

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2015

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ABSTRACT OF THESIS

AN INVESTIGATION INTO THE OCCURRENCE OF *BATRACHOCHYTRIUM DENDROBATIDIS* INFECTION IN PLETHODONTID SALAMANDER COMMUNITIES OF ROBINSON FOREST

Environmental and anthropogenic stressors negatively affect amphibians in a variety of ways, often increasing their vulnerability to pathogen infection and mortality. Sampling for the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd) was conducted in order to: 1) determine the presence of chytrid infection in stream-associated plethodontid salamanders of southeastern Kentucky, and 2) evaluate differences in infection intensity between salamanders residing in intact forest streams, timber-harvested streams and surface-mined streams. During 14 sampling sessions occurring between March, April and May of 2013, I collected DNA samples from 306 individual salamanders within 8 species from the family Plethodontidae; additional amphibians (i.e. frogs, newts) were opportunistically sampled when encountered. Approximately 2.1% of the salamanders and 50% of the frogs sampled from intact streams, 2.3% of the salamanders and 80% of the frogs sampled from harvested streams, and none of the salamanders and 100% of the frogs sampled from mined streams tested positive for Bd. No significant differences in occurrence of Bd or infection intensity were detected between the treatment sites ($\chi^2 = 0.59$; p-value = 0.75), or between individuals of a species from different treatment sites (see tables). These findings are the first to demonstrate that *B. dendrobatidis* is present in amphibians of eastern KY.

KEYWORDS: *Batrachochytrium dendrobatidis*, plethodontid salamanders, Appalachia, resource extraction, chytridiomycosis

Sarah H. Spaulding

June 10, 2015

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CHAPTER I

An investigation into the occurrence of *Batrachochytrium dendrobatidis* infection in plethodontid salamander communities of Robinson Forest

Amphibian Declines

Significant amphibian declines have been documented on a global level and at a highly accelerated rate over the past three decades (Stuart *et al.*, 2004; McCallum, 2007). Global population assessments indicate that amphibians are declining more rapidly than other vertebrates, and current amphibian extinction rates may be more than 200 times greater than background extinction rates (McCallum, 2007). More than 100 amphibian species have disappeared since 1980, and another 400 are rapidly declining and estimated to be on the verge of extinction. Unfortunately, data deficiency in over 20% of amphibian species has prevented the determination of their conservation status, and therefore numbers of imperiled species are most likely underestimated (Stuart *et al.*, 2004). Additionally, half of the 400 rapidly declining amphibian species are suffering enigmatic declines, meaning that the causes of their declines are not altogether understood or well established.

Habitat degradation is generally considered the primary threat to amphibian populations, though stressors such as pollution, invasive species, climate change, overexploitation, and novel pathogens are also contributing to the rapid decline of amphibians (McCallum, 2007). Climate change and disease are frequently suggested as the

most common causes of enigmatic declines, and it is plausible that stressors may synergistically exacerbate the disappearance of amphibian populations. Additionally, amphibians are known to host a wide range of microparasites (i.e. viruses, bacteria and fungi) and macroparasites (e.g. nematodes, mites and copepods) that can cause disease and mortality (Blaustein *et al.*, 2012). Parasites and pathogens do not always result in host-population or species-level mortality and have even been known to promote biodiversity by acting as indicators of ecosystem productivity and resilience. Evolutionarily, pathogen-induced die-offs are counterproductive, as they can result in reduced host transmission rates; however, some pathogens are remarkably deleterious to amphibian viability (e.g. *Batrachochytrium dendrobatidis*) and have been known to cause numerous population-level declines and species extinctions.

Chytridiomycosis

Berger *et al.* (1998) were the first researchers to propose *Batrachochytrium dendrobatidis* (Bd) as the pathogen responsible for sudden declines in amphibian populations for which there were no other obvious explanations. Using histological techniques, Berger *et al.* (1998) identified the infection of the previously unreported fungus (Bd) in the epidermis of sick and dying frogs from Central America and Australia. Bd is the first fungal pathogen discovered to parasitize vertebrates and, since its discovery, has been identified on every continent amphibians inhabit (Berger *et al.*, 1998; Blaustein *et al.*, 2012). A growing number of species are known to be vulnerable to Bd infection, and the fungus is now blamed for numerous global amphibian population declines and extinctions (Blaustein *et al.*, 2012; Berger *et al.*, 1998; Cheng *et al.*, 2011; Lips *et al.*, 2006; Longcore *et al.*, 2007; Muths *et al.*, 2003; Rosenblum *et al.*, 2010; Rovito *et al.*, 2009; Skerratt *et al.*, 2007). The fungus typically requires moisture and an optimal temperature between 17-25°C for

significant growth and development, though it has been observed growing and reproducing outside these optima, allowing for persistence in non-ideal environments (Piotrowski *et al.*, 2004). The aquatic nature and thermal preferences of Bd correlate with amphibian die-offs, which have been documented at cool, higher-elevation streamside locations (Blaustein *et al.*, 2012).

The life cycle of Bd is complex and consists of an infective, substrate-independent, aquatic zoospore stage as well as a non-motile, substrate-dependent zoosporangia stage (Piotrowski *et al.*, 2004). Zoospores are flagellated, capable of moving short distances, and chemotactically attracted to the various sugars, proteins and amino acids present on the amphibian epidermis (Moss *et al.*, 2008). Once a zoospore reaches an amphibian, it begins to digest superficial, keratinized epidermal cells of the animal (Piotrowski *et al.*, 2004). As it exploits the epidermis for nutrition, the zoospore encysts itself within the deeper epidermal cells of the amphibian and develops into zoosporangia. The rate of development into zoosporangia coincides perfectly with the maturation of amphibian epidermal cells (Berger *et al.*, 2005). Zoosporangia in the outermost epidermal layer then produce new zoospores, which are either discharged into the aquatic environment or the amphibian host, accelerating the progression and manifestation of the disease chytridiomycosis.

Larval infection is restricted to keratinized mouthparts, however post-metamorphic individuals may be infected over their entire body and exhibit epidermal hyperplasia, hyperkeratosis and abnormal skin sloughing as zoosporangia re-infect their host and the disease progresses (Rosenblum *et al.*, 2010). Larval infection may result in reduced growth or slowed development by impairing foraging abilities; however, infections in post-metamorphic individuals can have much more devastating results (Blaustein *et al.*, 2012). Severely infected individuals exhibit clinical symptoms such as increased thickening and

shedding of infected skin, lethargy, abnormal posture, anorexia, convulsions and an impaired righting reflex (Berger *et al.*, 1998; Berger *et al.*, 2005). These individuals eventually succumb due to the disruption of epidermal regulatory function, osmotic imbalance and electrolyte transport impairment resulting in cardiac arrest (Voyles *et al.*, 2007; Voyles *et al.*, 2009).

Amphibian Life History and Bd Infection

Susceptibility to infection and the manifestation of chytridiomycosis can vary greatly among different species, and there is even variation within individuals of the same species living in different regions or habitats (Moss *et al.*, 2008; Blaustein *et al.*, 2012). Amphibian species demonstrating rapid, Bd-associated declines typically exhibit a non-random topographical distribution, with declines occurring most frequently and rapidly in cool, higher-elevation tropical streams and often in well-protected areas such as national parks and nature preserves (Berger *et al.*, 1998; Lips *et al.*, 2006; Rovito *et al.*, 2009; Stuart *et al.*, 2004). Various studies suggest that amphibian populations in Central America, Caribbean and Australia have suffered the most devastating losses; however, chytridiomycosis is also infecting North American amphibians, where Ouellet *et al.* (2005) suggest that infection has been occurring since the 1960s. Longcore *et al.* (2007) showed that seven out of nine frog species sampled from the northeast United States tested positive for the fungus, and Bd has also been implicated in anuran population crashes in Arizona, California and Colorado (Bradley *et al.*, 2002; Briggs *et al.*, 2005; Muths *et al.*, 2003).

Bancroft *et al.* (2011) examined traits of 117 North American amphibian species and assessed their relationship to Bd infection. They found that size at maturity, egg-laying behavior and dependence on aquatic habitats correlate with, or predispose amphibians to Bd infection. Amphibians that mature at a larger size often live longer, and longevity may

increase the potential for infection. Additionally, larger amphibians have more surface area, which may increase infection potential, as more keratinized epidermal tissue is available to sustain the fungus. Species that oviposit in large clusters appear to be more susceptible to infection when compared to species that lay eggs singly or in small clusters. This may be due to the varying lengths of time different that different species physically interact during mating, as species that lay eggs in large clusters of eggs are likely to amplex or remain in contact for longer, effectively increasing the chance of transmission of zoospores between individuals. Finally, aquatic species appear to be more susceptible to infection than terrestrial species, which is unsurprising considering the aquatic nature of the fungus.

While tropical frog populations have, thus far, been the most detrimentally affected by Bd, the incredible diversity of North American salamanders may be at risk from this disease, as Bd infection has been documented in a variety of salamander species from the eastern United States, Texas, and Mexico (Gaertner *et al.*, 2009; Van Rooij *et al.*, 2011; Hossack *et al.*, 2010; Gratwicke *et al.*, 2011; Souza *et al.*, 2012; Chatfield *et al.*, 2009; Byrne *et al.*, 2008; Rothermel *et al.*, 2008; Rollins *et al.*, 2013; Hossack *et al.*, 2010; Gratwicke *et al.*, 2011). The greatest concentration and diversity of North American salamanders are located in the Appalachian region, where they comprise a significant amount of trophic biomass and are the dominant vertebrate predators (Davic & Welsh, 2004). The forest ecosystems of this region have suffered enormously due to the pollution, sedimentation and habitat degradation caused by various resource extraction practices (e.g. surface mining, timber harvest). Environmental degradation may increase susceptibility to pathogen infection and mortality in amphibians by compromising the immune systems of local amphibians (Blaustein *et al.*, 2012).

Resource Extractive Impacts

Factors such as pollutants, climate change, habitat alteration and increases in UV radiation are known to cause an immunocompromised condition in various amphibian species by affecting body condition, water and electrolyte balance, endocrine regulation, metabolism, behaviors, and pathogen replication rates (Blaustein *et al.*, 2012; Voyles *et al.*, 2007). However, environmental stressors may cause both an increase in vulnerability to disease in amphibians, as well as a decrease in the likelihood of transmission of between individuals due to a reduction in host density (Lafferty & Holt, 2003). Some stressors may also benefit hosts, to the detriment of their pathogens. For example, pollutants and contaminants may be more toxic to the parasite or pathogen than they are to the host, and the free-living, motile Bd zoospore is vulnerable to a variety of pesticides and fungicides (Hanlon & Parris, 2012).

A recent survey by St-Amour *et al.* (2008) found no relationships between the prevalence of chytrid infection in Canadian ponds and distance to a variety of anthropogenic habitat modifications (e.g. buildings, roads, agricultural activity). The effects of resource extraction processes in Appalachia on amphibian health remain insufficiently studied, and these practices may affect amphibian health and communities differently than the presence of roads or farms do. For example, surface mining activities typically result in mine spoil (overburden material) being pushed into adjacent valleys that creates a valley fill characterized by burial of major portions of ephemeral, intermittent and perennial streams. Streams emerging from the base of valley fills typically exhibit decreased vertebrate and invertebrate biodiversity, elevated levels of total dissolved solids, greater specific conductivity, and altered pH levels relative to undisturbed streams (Fritz *et al.*, 2010; Palmer *et al.*, 2010; Barton, 2011; Lindberg *et al.*, 2011; Muncy *et al.*, 2014).

Timber harvests can also compromise the health of local stream-associated amphibian populations, as harvesting has been shown to cause an increase in turbidity, total dissolved solids, and sedimentation within affected streams (Witt *et al.*, 2013). An increase in sedimentation can be particularly detrimental, as it can result in the reduction or elimination of areas in the streambed substrate associated with refugia and feeding in larval, stream-associated amphibians (Witt *et al.*, 2013; Lowe and Bolger, 2002; Wilson and Dorcas, 2003; Maigret *et al.*, 2014). Recently, Maigret *et al.* (2014) and Muncy *et al.* (2014) demonstrated that the abundances of several species of salamander are negatively affected by timber harvest and surface mining, respectively, in Robinson Forest; however, questions remain about whether and how Bd might be impacting salamanders in these areas affected by anthropogenic resource extraction.

Research Objectives

In spring 2013, I conducted research to determine whether Bd was present in one of the more isolated and mature mixed-mesophytic forest tracts of southeastern Kentucky, and compared infection intensity and prevalence to nearby sections of this forest impacted by timber harvest and surface mining, two regionally common extractive activities. I focused on sampling individuals within the Plethodontidae (lungless) salamander family, as Bd-induced population declines have already been documented in individuals of this family within Mexico and Central America (Lips *et al.*, 2006; Rovito *et al.*, 2009; Cheng *et al.*, 2011). I hypothesized that salamanders inhabiting streams impacted by surface mining and timber harvest would have a higher prevalence of Bd than those inhabiting streams in mature forest. At the time of this study I was unaware of any published findings on chytrid infection in salamanders anywhere in Kentucky, or of any studies that attempted to compare differences in infection prevalence between forest streams in mature forest with

those impacted by said resource extraction practices. It is my hope that findings from my study will prove useful to our knowledge about where Bd occurs and what factors may be important in determining the likelihood of infection.

METHODS

Study Sites

Samples were collected from low-order streams within University of Kentucky's Robinson Forest, located in southeastern Kentucky within Breathitt, Knott and Perry counties. Robinson Forest (RF) is a second growth, mixed mesophytic system that is used as an extension, education and research facility by the Forestry Department at the University of Kentucky. The forest consists of approximately 15,000 acres that are distributed among eight fragmented sections, with the main block comprising approximately 10,000 acres encompassing the Clemons Fork and Coles Fork watersheds, both of which are considered reference streams by the Environmental Protection Agency. The plant assemblages in this forest are dominated by species including hickory (*Carya* spp.), sugar maple (*Acer saccharum*), red maple (*Acer rubrum*), sassafras (*Sassafras albidum*), tulip poplar (*Liriodendron tulipifera*), northern red oak (*Quercus rubra*), chestnut oak (*Quercus montana*), white oak (*Quercus alba*), black walnut (*Juglans nigra*), yellow buckeye (*Aesculus flava*), eastern hemlock (*Tsuga canadensis*), Virginia pine (*Pinus virginiana*) and eastern white pine (*Pinus strobus*) (Overstreet, 1984; R. Paratley, University of Kentucky, personal communication).

The University of Kentucky acquired Robinson Forest in 1923 after it had been extensively logged, and the majority of RF now consists of 90 year-old second-growth

forest. Samples from the control and harvest treatments were obtained from randomly selected low-order streams within the Clemons Fork watershed. Nine streams were randomly selected for sampling within the control treatment, which consists of the aforementioned 90 year-old second-growth mesophytic forest. Between 2008-2009, six headwater streams were logged within Clemons Fork using a two-aged deferment harvest, in accordance with one of three separate SMZ treatments (Witt *et al.*, 2013; Maigret *et al.*, 2014). I randomly chose eight streams within one randomly selected streamside management zone configuration to use as our harvested treatment sites; this treatment consisted of streams harvested with a mandatory retention of channel bank trees, limited equipment tracking, and improved stream crossings (Witt *et al.* 2013). In addition to collecting DNA samples from control and harvested streams within the Clemons Fork watershed, I also collected samples from low-order streams located within the Laurel Fork watershed, a 2200-acre section of RF located approximately 5km southwest of the main block. During the 1990s, a large portion of the Laurel Fork watershed was surface mined for coal, and as a result many of the existing stream networks were buried under valley fills. Five low-order streams in the Laurel Fork watershed were randomly selected for sampling within the mined treatment, and sampling areas for the control, harvested and mined sites were approximately equivalent in size. Detailed maps of the study sites and sampled streams can be found in Figures 1.1, 1.2 and 1.3.

Sampling Methods

Sampling sessions were conducted in March, April and May of 2013, when air temperatures were relatively cool and average relative humidity high. Temperature and precipitation data for sampling days can be found in Appendix A. Sampling during these environmental conditions increases the chances of Bd detection due to its environmental

and temperature optima (Piotrowski *et al.*, 2004; Kriger and Hero, 2007). A minimum of 20 individuals of a given species, per stream, provided a sufficient sample size to obtain a 95% probability of detecting Bd for a given species within the stream (Cameron & Baldock, 1998). This study focused on collecting DNA samples from salamanders within the family Plethodontidae; however, samples were obtained from other amphibians (*i.e.* frogs, newts) when encountered. Starting at 20m upstream from where the stream joined an intermittent or perennial stream, and continuing upstream until physical access to the stream was impossible, each stream was sampled once by turning over logs and rocks within or near the water. Leaf litter searches were also conducted within 3m of the stream bank.

Great care was taken during the handling of individual salamanders, as well as when traveling between sites, in order to minimize the chance of cross-contamination and injury to the animals. A minimum of two individuals were present during each sampling event; one individual was responsible for measuring the amphibians, collecting DNA samples, and recording data, while the other(s) looked for and captured the amphibians. Disposable nitrile gloves were used when handling amphibians, as they seem to be relatively non-toxic to amphibians and they kill Bd on contact (Mendez *et al.*, 2008). Gloves were replaced after each individual was captured and sampled, and boots and equipment were sterilized with bleach after sampling was completed at each stream. Each captured amphibian was restrained by placement in a clear plastic Ziplock bag before being identified and examined visually for gross abnormalities characteristic of Bd infection (*e.g.* lesions or sloughing skin), then released. DNA samples were obtained by using a sterile, individually wrapped cotton-tipped swab to rub the limbs, in between the digits, and the ventral surface of the salamander 20 times in accordance with Hyatt *et al.* (2007). Swabs were placed into individual tubes filled with ethanol, and were then labeled and stored at 4°C until they were shipped to colleagues in an external lab for molecular analysis.

DNA Analysis

Quantitative polymerase chain reaction (qPCR) was used to determine the presence and quantity of *Batrachochytrium dendrobatidis* (Bd) DNA in the samples. This method is highly sensitive, as qPCR can detect as little as one zoospore in a sample and, using a standard curve, can provide an estimation of pathogen load by comparing samples to a set of standards (Kerby *et al.*, 2013). DNA was extracted from the swabs using the Qiagen DNeasy kit following the manufacturer's procedures eluted into 100µL of buffer. For the qPCR, 20µL reactions containing 1X Taqman Gene Expression Master Mix, 900nmol 5.8SChytr Primer, 900nmol ITS-1Chytr Primer, 250nmol Chytr MGD Probe, DEPC water and 5µL of extracted DNA template were used. Plates were run on a StepOne qPCR machine (Life Technologies) at an activation stage of 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Each plate included a negative control and standard curve from 1.573 – 1.573*10e6 molecules/µL, with an estimation of 7 molecules per zoospore (standards provided by Pisces Molecular). Pooled samples were run in triplicate, with five individuals per pool sample. Any pooled samples with >2 replicates amplified with cycle threshold (Ct) values <40 were marked as positive. The positive pools were then run in a separate plate as individual samples, in triplicate. StepOne software v2.2.2 (Applied Biosystems) was used to quantify Bd loads from the qPCR results.

Statistical Analysis

R (version 3.1.0) was relied upon for data analysis. A Chi-square test was used to determine whether there was a significant difference in the proportion of infected individuals between watershed treatments. This test utilizes “contingency tables” to

generalize relationships between the explanatory variable (treatment) and the response variable (Bd infection), where

$$\chi^2 = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

and the null hypothesis assumes that the proportion of infected individuals does not significantly differ between treatment sites ($H_0: p_1 = p_2 = p_3$). Large, positive values of χ^2 contradict the null hypothesis and indicate that it should be rejected. Chi-square analysis was also used to determine if there were any differences between the proportions of infected individuals within a particular species from different treatment sites. The number of individuals testing positive for the fungus was insufficiently large enough to confidently compare infection intensity between sites.

RESULTS

During the fourteen sampling sessions I sampled a total of 324 amphibians (307 salamanders and 17 frogs). Two hundred samples (61.7%) were obtained from control streams, 93 (28.7%) from timber harvested watersheds, and 31 (9.6%) from surface mine-impacted streams (Table 1.1). All salamanders captured and sampled were individuals from 8 species of family Plethodontidae, with the exception of one juvenile red-spotted newt (*Notophthalmus viridescens*, family Salamandridae). All 17 frogs opportunistically captured and sampled were individuals from 2 species within the family Ranidae.

Although none of the sampled amphibians exhibited clinical symptoms of chytridiomycosis, Bd was detected on 17 of 324 (5.2%) amphibians captured across all watersheds. By watershed, this included 9 of 200 (4.5%) amphibians from control streams (specifically 4 [2.1%] salamanders and 5 [50%] frogs), 6 of 93 (6.5%) amphibians from

timber harvested watersheds (specifically 2 [2.3%] salamanders and 5 [80%] frogs), and 2 of 31 (6.5%) amphibians from the surface-mined watershed (specifically 2 [100%] frogs) (Table 1.1; Figures 1.4, 1.5 and 1.6). Chi-square analysis revealed no significant differences in the proportion of individuals infected between treatments sites ($X^2 = 0.586$, $p\text{-value} = 0.764$; Appendix A). Additionally, there were no significant differences detected between the proportions of infected salamanders of affected species between control and harvest treatments (*e.g.* proportion of *D. fuscus* testing positive within control streams vs. proportion of *D. fuscus* testing positive within harvested streams; Appendix A). For each treatment, the number of infected individuals was insufficiently large enough to confidently compare the intensity of infection between sites. The positive samples collected from the Plethodontid salamanders yielded between 0.42 – 25.66 Bd DNA molecules (or 1 – 4 zoospore equivalents), and the positive samples collected from the ranid frogs yielded between 2.02 – 5697.80 Bd DNA molecules (or 1 – 814 zoospore equivalents) (Table 1.2).

DISCUSSION

While no significant differences were found in the occurrence and infection intensity of Bd from individuals inhabiting the different extractive resource treatments, this study is the first to document Bd infection in amphibians of this highly biodiverse forested region of Kentucky. Although Bd was not detected in salamanders captured in the surface mined watershed, the possibility of infected salamanders being present cannot be ruled out due to difficulty in finding amphibians to sample and resultant small sample size ($n = 29$), and the fact that samples collected from cohabitating ranid frogs within these mined streams tested positive for Bd.

Hypothetically, environmental stressors could increase susceptibility to Bd infection and the manifestation of chytridiomycosis in uninfected individuals, as stressed individuals often lack the requisite energy to successfully defend themselves against pathogen infection and disease progression (Lafferty & Holt, 2003). Although I determined that Bd is present in the surface mined watershed, the low level of Bd detection may be attributed to several factors. For example, if the stressors that individuals are exposed to also reduce factors such as available resources, reproductive rate, survivorship and population density, then disease transmission may be reduced concomitantly via a density-dependent reduction of exposure and contact between infected hosts and uninfected individuals. Sampling within the mined streams occurred latest in the spring, and elevated ambient temperatures may have influenced our ability to detect the fungus on the salamanders I encountered (see Appendix B for air temperature and rainfall data).

Temperatures $>25^{\circ}\text{C}$ cause both an increase in the rate of skin shedding in amphibians and a reduction in growth and development of Bd (Piotrowski *et al.*, 2004). Elevated rates of skin cell shedding may result in a diminished abundance of Bd zoosporangia within the superficial epidermis of amphibians; however, due to the fossorial, semi-aquatic or aquatic nature of native Plethodontid salamanders, it is likely that the refugia they seek out during warmer days are located in moist, cool microhabitats that are suitable to the survivorship and reproduction of Bd. Regardless, Kriger & Hero (2007) recommend that chytrid surveys occur when mean air temperatures are between 12 and 19 $^{\circ}\text{C}$, when chytrid infections are most likely to be detected, and maximum temperatures for the days during which the mined sites were sampled exceeded these recommended temperatures.

Bd typically thrives in environments with neutral pH, and streams altered by mining and valley fills generally have an alkaline pH due to exposure of the stream water to carbonate minerals (Palmer *et al.*, 2010). The mined streams sampled for this study appear to be no exception, as Muncy *et al.* (2014) detected elevated pH levels in the mined streams of the Laurel Fork watershed while investigating the effects of mountaintop removal mining and valley filling on salamander occupancy and community structure. However, while non-neutral pH levels may result in a decreased abundance of free-living aquatic Bd zoospores, the pH levels of the mined streams in this study were nonetheless suitable for the survivorship of Bd and, as such, it is unlikely that this was a factor in the low levels of Bd detection in mined streams (Piotrowski *et al.*, 2004). It may be that some other water quality parameter (*e.g.* turbidity, dissolved oxygen levels, etc.) may be negatively affecting Bd zoospore levels.

Salamander detection and capture for my study did occur less often in mined and timber-harvested streams than in sampled control streams (*i.e.* 29 and 88 vs. 190, respectively). This is unsurprising, as these resource extraction practices have recently been shown to negatively affect the abundance and occupancy of various stream-associated salamander species living within streams impacted by these activities (Maigret *et al.*, 2014; Muncy *et al.*, 2014). Finally, the species composition in the mined streams appeared to vary from that of the harvested and control streams; specifically, 3 of the 4 species (*i.e.* *Desmognathus monticola*, *Eurycea cirrigera* and *Pseudotriton ruber*) in which Bd was detected in the control and harvested streams were not encountered during sampling of the mined streams.

It should also be reiterated that life history characteristics might affect the potential of an individual or species to be vulnerable to Bd infection (Bancroft *et al.*, 2011). For

example, many frogs exhibit amplexus mating behavior, in which a male physically embraces a female, sometime for days, during external fertilization (Wells, 2007). While most salamanders do not embrace in amplexus, many Plethodontid salamanders have complex courtship rituals involving prolonged chemical and tactile stimulation between male and female individuals (*e.g. Pseudotriton ruber, Desmognathus monticola*), and this may make them more vulnerable to transmission than salamanders that do not engage in this type of courtship behavior (Brock & Verrell, 1994).

Additionally, whether an individual is terrestrial, semi-aquatic, or aquatic may influence their susceptibility to infection (Bancroft *et al.*, 2011). For example, the ranid frogs in which I detected Bd infection (*Lithobates clamitans* and *L. palustris*) are relatively aquatic and often abundant in permanent fresh waters (Wells, 2007). The same is true for the Plethodontid salamanders that tested positive for the fungus (*i.e. Desmognathus fuscus, D. monticola, Eurycea cirrigera* and *Pseudotriton ruber*). In contrast, most of the salamanders that tested negative for Bd are more terrestrial and prefer the moist, wooded slopes of valleys and ravines. (*e.g. Plethodon glutinosus, P. richmondi, Eurycea longicauda*). Interestingly, none of the individuals of most aquatic salamander species I encountered and sampled (*i.e. Gyrinophilus porphyriticus*) tested positive for the fungus. I found this to be particularly surprising, as *G. porphyriticus* is highly aquatic and cannibalistic and frequently predated upon smaller individuals of species that we found to be infected (Bruce, 1979).

Potentially low zoospore levels in mined stream water, differences in salamander life-history characteristics, and decreased occupancy of salamanders along with the change in encountered species compared with harvested and control streams, may help to explain why none of the salamanders sampled from the mined streams tested positive for Bd infection. In general, Bd appears to be present in low levels in Robinson Forest, and none of

the individuals that tested positive for Bd infection exhibited clinical symptoms of disease progression. While our sample size of infected individuals was insufficiently large enough to confidently compare infection intensity between species occupying watersheds impacted by resource extraction, the ranid frogs encountered generally appeared to be more heavily infected than salamanders. Regardless, this study has documented Bd infection in both Plethodontid salamanders and Ranid frogs of eastern Kentucky.

In general, my findings are similar to other chytrid surveys conducted in the Appalachian region that suggest a geographically widespread pattern of subclinical chytrid infection, with the prevalence and intensity of infection in frogs apparently greater relative to that of salamanders (Rothermel *et al.*, 2008; Rollins *et al.*, 2013; Hossack *et al.*, 2010; Gratwicke *et al.*, 2011, Muletz *et al.*, 2014). Martel *et al.* (2013) have recently identified a new species of chytrid fungus in Europe (*Batrachochytrium salamandrivorans* [Bs]) that exclusively parasitizes salamanders, and that has been shown in a lab setting to be lethal to several North American species (Martel *et al.*, 2014). Going forward, I recommend widespread sampling of Kentucky amphibians in order to determine the occurrence of Bd and Bs infection in our highly biodiverse and amphibian-rich state so as to better understand and perhaps manage for these fungal diseases that have been implicated in the global loss and decline of many amphibian species.

Table 1.1. Bd infections organized by species and watershed treatment for amphibians captured between 13 March and 1 May 2013 from Clemons Fork and Laurel Fork watersheds, Robinson Forest, Kentucky. The number of individuals testing positive for Bd, per species for each treatment, can be found within parentheses, followed by the percentage testing positive, per species for each treatment. Salamander data are presented first, data for the two frog species encountered are given last.

Species	Treatment			
	Control	Harvest	Mined	Total
<i>D. fuscus</i>	55(1) 1.8%	20(1) 5%	4(0)	79(2) 2.5%
<i>D. monticola</i>	66(1) 1.5%	38(1) 2.6%	-	104(2) 1.9%
<i>D. spp</i>	6(0)	-	-	6(0) 0%
<i>E. cirrigera</i>	35(1) 2.8%	8(0)	-	43(1) 2.3%
<i>E. longicauda</i>	-	-	7(0)	7(0) 0%
<i>G. porphyriticus</i>	10(0)	9(0)	-	19(0) 0%
<i>P. glutinosus</i>	-	3(0)	1(0)	4(0) 0%
<i>P. richmondi</i>	16(0)	10(0)	16(0)	42(0) 0%
<i>P. ruber</i>	2(1) 50%	-	-	2(1) 50%
<i>N. viridescens</i>	-	-	1(0)	1(0) 0%
Total (salamanders)	190(4) 2.1%	88(2) 2.2%	29(0) 0%	307(6) 1.7%
- - - - -				
<i>L. clamitans</i>	10(5) 50%	5(4) 80%	1(1) 100%	16(10) 62.5%
<i>L. palustris</i>	-	-	1(1) 100%	1(1) 100%
Total (frogs)	10(5) 50%	5(4) 80%	2(2) 100%	17(11) 64.7%
- - - - -				
Total (all captures)	200(9) 4.5%	93(6) 6.5%	31(2) 6.5%	324(17) 5.2%

Table 1.2. *Batrachochytrium dendrobatidis* DNA molecule quantity and equivalent zoospore load per swab sample, organized by species and treatment. Swab sample analyses were run in triplicate (Q I, II, III), and the average quantity of DNA molecules per sample (Avg Q) is provided alongside individual run data. The quantity is the amount of DNA estimated to be present based on DNA plasmid standards provided by Pisces Molecular. The zoospore equivalents are also based on information provided by Pisces Molecular, which estimates an average of 7 DNA molecules per zoospore. Swab zoospore loads are provided parenthetically in decimal values, as well as whole number zoospore equivalents.

Treatment	Organism	Q I	Q II	Q III	Avg Q	Zoospore Equivalent
Control	<i>D. monticola</i>	2.52	2.92	0.711	2.05	(0.293) 1
	<i>D. fuscus</i>	19.1	32.4	25.4	25.7	(3.67) 4
	<i>E. cirrigera</i>	4.36	2.44	2.01	2.94	(0.42) 1
	<i>P. ruber</i>	1.24	2.28	3.45	2.32	(0.33) 1
	<i>L. clamitans</i>	8.10	10.3	11.5	9.98	(1.43) 2
	<i>L. clamitans</i>	34.9	32.5	36.0	34.5	(4.92) 5
	<i>L. clamitans</i>	9.35	14.5	11.2	11.7	(1.67) 2
	<i>L. clamitans</i>	1.50	2.75	5.49	3.25	(0.46) 1
	<i>L. clamitans</i>	802	890	949	880	126
Harvest	<i>D. monticola</i>	0.579	n/a	0.265	0.42	(0.06) 1
	<i>D. fuscus</i>	1.72	1.53	2.13	1.79	(0.256) 1
	<i>L. clamitans</i>	5890	5530	5674	5698	814
	<i>L. clamitans</i>	3.37	2.30	0.398	2.02	(0.289) 1
	<i>L. clamitans</i>	634	590	685	636	(90.9) 91
	<i>L. clamitans</i>	208	211	197	205	(29.3) 30
Mined	<i>L. clamitans</i>	6.08	1.46	4.95	4.16	(0.594) 1
	<i>L. palustris</i>	57.7	55.8	73.7	62.4	(8.91) 9

Figure 1.1. Map of streams sampled within Clemons Fork and Laurel Fork watersheds. Control streams are represented in black, timber-harvested streams are represented in red, and surface-mined streams are represented in yellow.

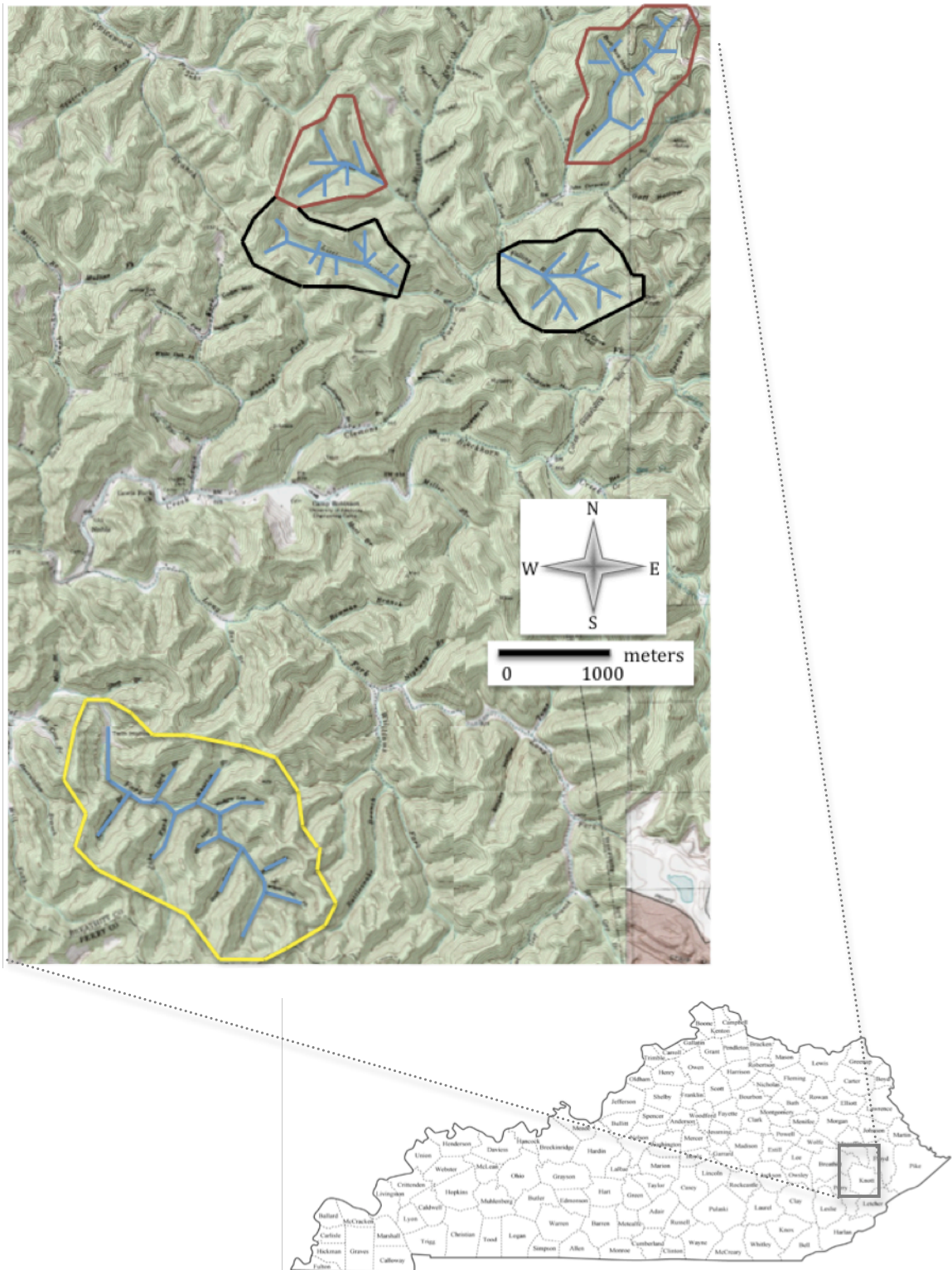


Figure 1.3. Map detail of Laurel Fork watershed sampling sites. The five streams sampled from the surface-mined watershed are represented in yellow.

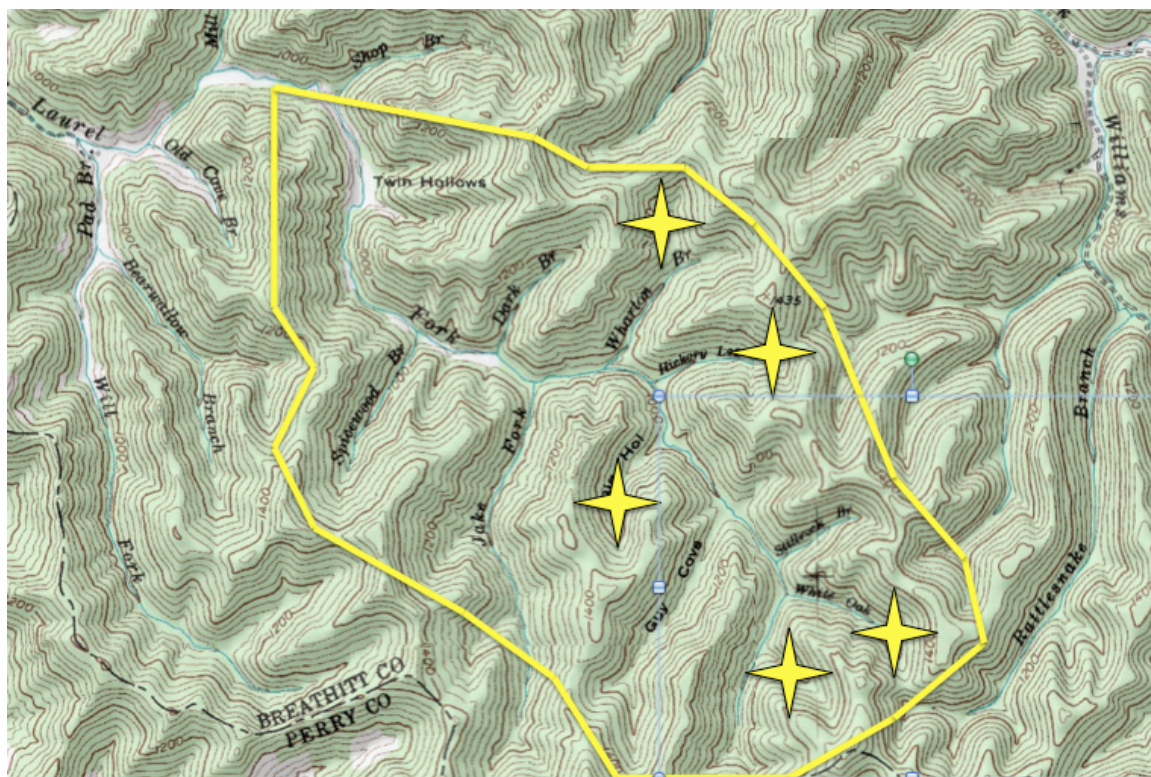


Figure 1.4. Amphibian captures organized by treatment and species. Gray bars indicate number of individuals, of a given species, which tested positive for Bd.

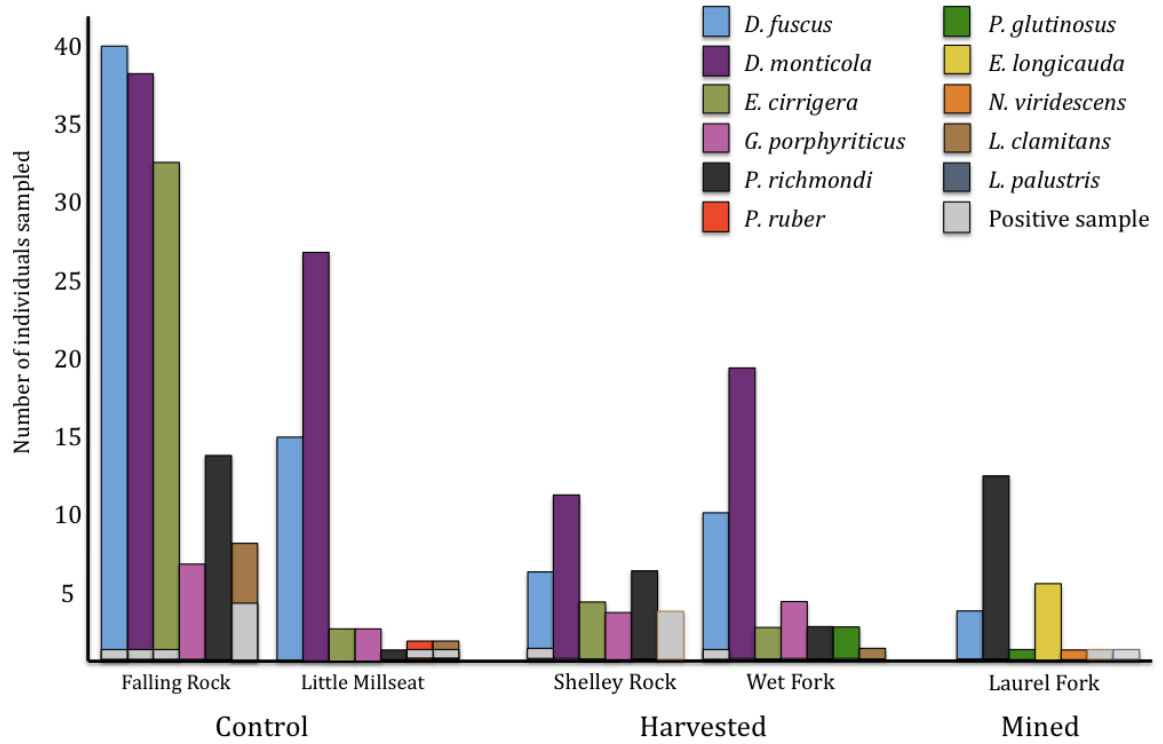


Figure 1.5. Bd detection organized by amphibian type and watershed treatment. Control streams are enclosed within the black areas; timber-harvested streams are enclosed within the red areas. Red circles indicate positive salamander samples; green circles indicate positive frog samples.

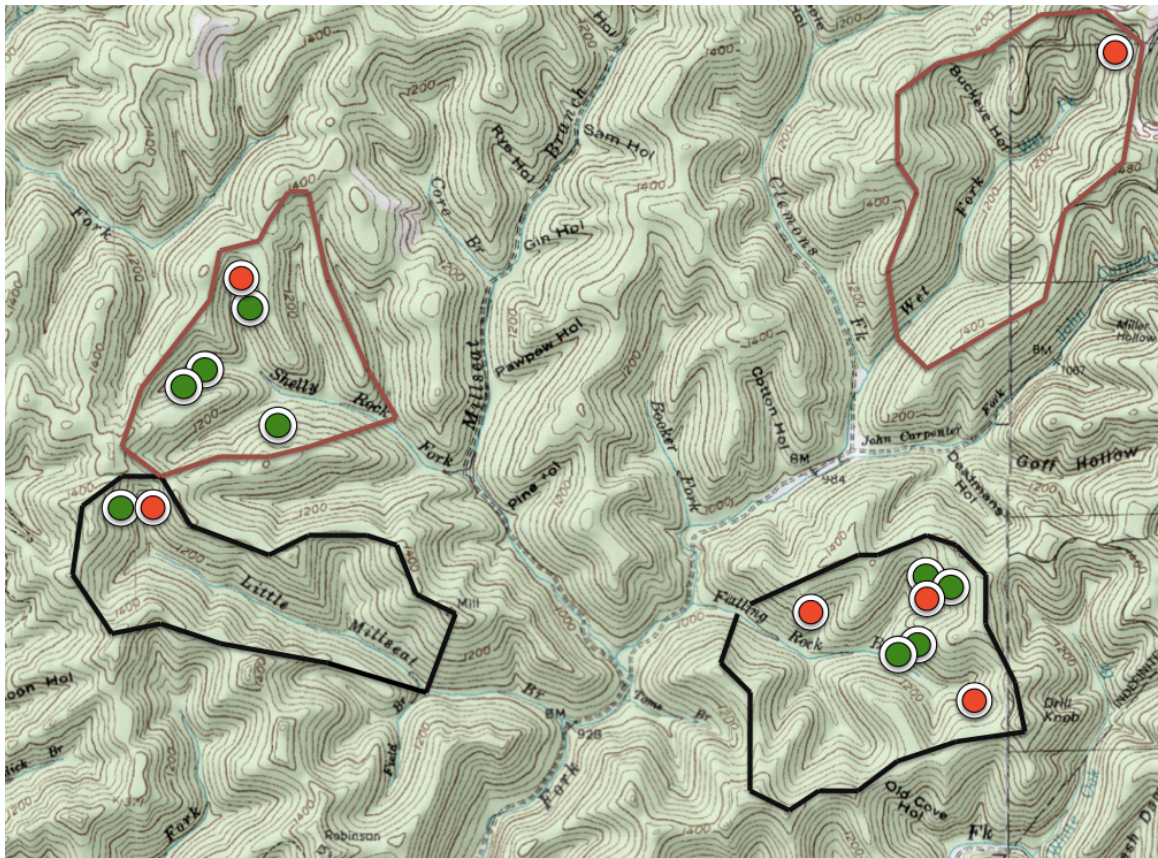
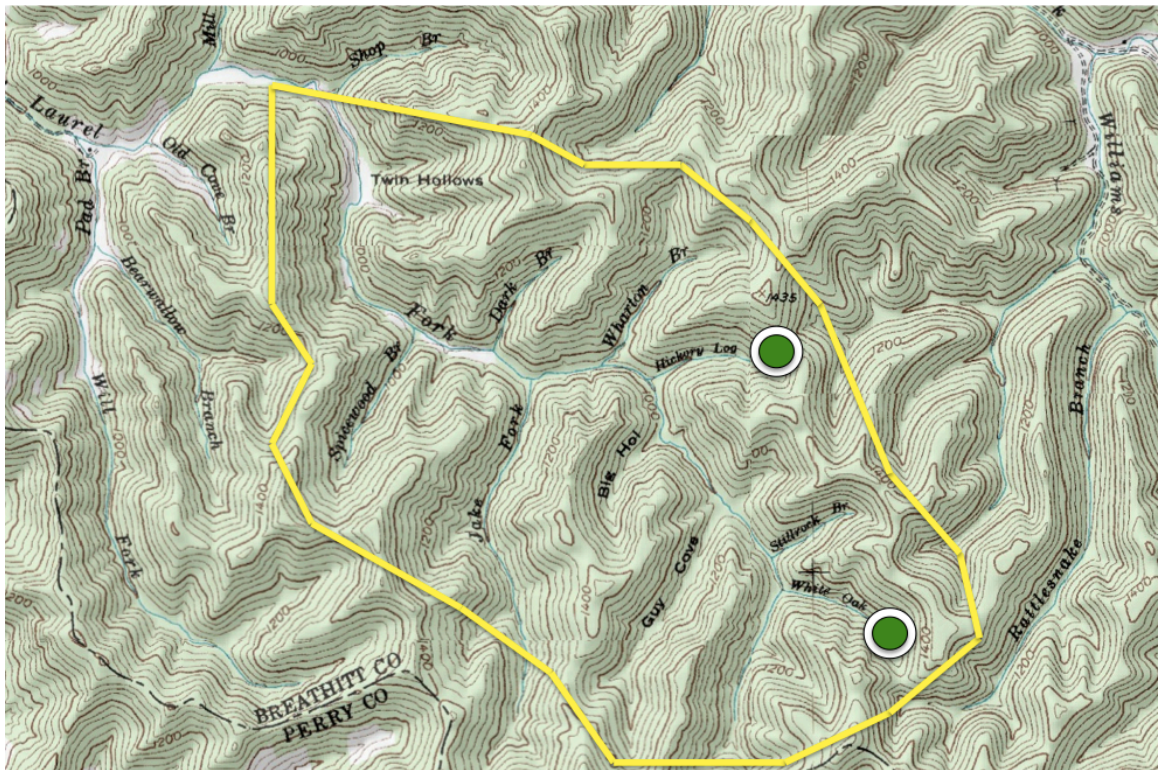


Figure 1.6. Bd detection organized by amphibian type and watershed treatment. Surface-mined streams are enclosed within the yellow areas. Green circles indicate positive frog samples; none of the salamanders encountered and sampled within the surface-mined streams tested positive for Bd.



Appendix A. Chi-square analysis of the difference in proportion of infected individuals between all treatments (a), as well as the difference in proportion of infected individuals of specific salamander species between control and harvested streams (b).

	(a)	(b)				
	All treatments	Control/Harvest				
	Total	<i>D. fuscus</i>	<i>D. monticola</i>	<i>E. cirrigera</i>	<i>L. clamitans</i>	Total
χ^2	0.586	0.572	0.159	0.234	1.25	0.177
p-value	0.746	0.449	0.690	0.629	0.264	0.674
df	2	1	1	1	1	1

Appendix B. Temperature and precipitation data for Robinson Forest for sampling days in March, April and May 2013. Temperature data are given in Fahrenheit, and parenthetically in Celsius.

	Air Temperature °F (°C)				Precipitation Total (in.)
Date	Max		Min		
3/13	37.4	(3.0)	29.5	(-1.4)	0.06
3/14	47.0	(8.3)	22.1	(-5.5)	0.00
3/15	63.9	(17.7)	26.0	(-3.3)	0.00
3/28	51.7	(10.9)	28.1	(-2.2)	0.00
4/2	52.4	(11.3)	25.8	(-3.4)	0.00
4/4	52.2	(11.2)	32.5	(0.28)	0.00
4/6	73.2	(22.9)	30.8	(-0.7)	0.00
4/7	74.5	(23.6)	38.8	(3.8)	0.00
4/8	76.2	(24.6)	43.7	(6.5)	0.00
4/9	82.2	(27.9)	50.2	(10.1)	0.00
4/10	86.9	(30.5)	48.8	(9.3)	0.00
4/25	63.4	(17.4)	38.7	(3.7)	0.17
4/30	80.5	(26.9)	44.4	(6.9)	0.02
5/1	80.2	(26.8)	49.4	(9.7)	0.00

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